EVALUATING THE EFFECT OF BODY FLUID MIXTURE ON THE RELATIVE EXPRESSION RATIO OF BLOOD-SPECIFIC RNA MARKERS

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Highlights:

- Blood-specific RNAs exhibited high expression levels in fresh pure bloodstains
- Blood-specific RNAs showed low or no expression in pure saliva and semen samples
- RERs of blood-specific RNAs to *U6* were not affected by body fluid mixture
- Some RERs were altered in mixed stains compared to pure bloodstains

Abstract

The estimation of the time elapsed since a biological stain was deposited at a crime scene can provide crucial information to a forensic investigation, indicating either when a crime was committed, or whether the biological evidence was deposited at the time of a known crime event. This would enable the investigators to limit the number of suspects and to assess alibis. The relative expression ratios (RERs) of body fluid-specific RNA markers are promising molecular tools for indicating the age of biological stains. However, the nature of some forensic samples found at crime scenes could be challenging, as they frequently occur in a mixture of different body fluid types. The research presented here has utilised reverse transcription quantitative PCR (RT-qPCR) to explore the impact of bloodstains being present in mixtures with other body fluids (saliva or semen) on the resulting RERs of blood-specific markers. The expression level of three blood-specific markers (*HBA*, *HBB* and *miR16*) along with two reference genes (*18S* and *U6*) were analysed across multiple ageing time points in pure and mixed bloodstains. For some markers, no significant differences were found when comparing RERs in pure and mixed bloodstains, however some RERs were altered in mixed stains. This indicates that the presence of body fluid mixtures may have a significant effect on the RERs of some blood-specific markers. This should therefore be considered when selecting markers for estimating the age of stains, particularly when multiple body fluids are thought to be present.

Keywords

Introduction

Determining the time since deposition of a biological stain at a crime scene can provide crucial information to a forensic investigation, indicating either when a crime was committed, or whether the biological evidence was deposited at the time of a known crime event. Previous studies have indicated that the relative expression ratio (RER) of reference genes (*ACTB*/*18S*) can be used to indicate the age of blood [1, 2], saliva [3] or hair samples [4]. However, it has also been suggested that there are limitations to this method when applied to samples that are mixtures of more than one type [3]. The RERs of body fluid-specific RNA markers have also been shown to be a potential method for estimating the age of body fluid stains, or the time since deposition [5, 6]. However, the nature of some forensic samples found at crime scenes could make this challenging, as they frequently occur in a mixture of different body fluid types. For instance, in a physical assault there could be a mixture of blood and saliva, or in sexual assaults there may be a mixture of semen and blood or saliva. Therefore, in order to develop such a method as a successful approach to estimating the age of biological stains in forensic casework samples, it is important that the effect of body fluid mixtures on RERs is evaluated.

In the context of forensic applications, a variety of RNA types have been identified as blood-specific markers, including the messenger RNA molecules Haemoglobin Subunit Alpha (*HBA*), and Haemoglobin Subunit Beta (*HBB*), which are protein subunits of the haemoglobin molecule, and the micro RNA *miR16*, which is involved in gene expression regulation [7-10]. These RNA molecules have been shown to degrade at different rates in bloodstains, with *miR16* marker exhibiting strong stability [5], likely due to its small size (~22 nucleotides) [11]. Interestingly, the RERs of these blood-specific markers have been shown to be positively correlated with the age of bloodstains [5], indicating that they may be reliable in estimating the time since deposition of bloodstains.

The aim of this research was to assess the effect of body fluid mixtures on the RER of blood-specific markers in samples stored over a period of two months. The expression levels of *HBA*, *HBB*, and *miR16* were analysed across multiple ageing time points in pure and mixed bloodstains using RT-qPCR. The RERs were calculated and compared

for pure blood samples and mixed samples, to determine whether there were any differences, and whether the RERs of blood-specific markers are over- or underestimated in mixed samples.

Materials and Methods:

Sample collection:

A total of 12 volunteers (6 males and 6 females) donated the blood, saliva and semen samples used in this study. Samples were collected from volunteers using procedures approved by the Departmental Ethics Committee in the Department of Pure and Applied Chemistry at the University of Strathclyde. Signed consent sheets were obtained from each donor after they had read a Participant Information Sheet (PIS). Two sample groups were set up as described below:

Pure and fresh body fluid samples:

This group of samples consisted of fresh body fluid stains (day = 0, *i.e*. not aged) from single body fluids (*i.e*. pure stains). Four volunteers donated blood samples in duplicate. The blood samples (20 µL) were collected onto sterile cotton swabs using disposable Unistik 3 comfort lancets. Another eight volunteers donated saliva and semen samples in duplicate (four donors for each body fluid). The samples were deposited into sterile collection pots. 20 µL of each sample was pipetted onto swabs and allowed to dry at room temperature before RNA extraction. Samples were collected using cotton swabs, as this is the most common technique for body fluid collection, whether from a crime scene or as trace evidence recovered from suspects or victims. Cotton swabs also have the benefit that they are easy to use and process, have guaranteed sterility, and are suitable for long-term storage.

Pure aged body fluid samples:

From the same volunteers, pure blood, saliva and semen samples (i.e. pure stains) were prepared in duplicate to be stored in a dark, dry place at room temperature, to simulate natural ageing until they reached a series of desired ageing time points (10, 30 and 60 days), at which stage total RNA was extracted.

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<u>Mixed body fluid samples:</u>

The second group of samples consisted of bloodstains mixed either with saliva samples or with semen samples. Mixtures were prepared by adding 20 µL of fresh blood from one volunteer to 20 µL of fresh saliva/semen from another volunteer on cotton swabs. The samples were all prepared in duplicate from 12 donors (*i.e.* each mixture sample consisted of stains from two donors). All the mixture samples were stored at room temperature in a dark, dry place to simulate natural ageing until they reach a series of desired ageing time points (0, 10, 30 and 60 days), at which stage total RNA was extracted.

RNA extraction:

Total RNA was extracted using the TRI® Reagent method (Sigma-Aldrich, Gillingham, UK) [1]. Genomic DNA was digested with the TURBO DNA-free™ Kit (Applied Biosystems, Life Technologies, UK) following the manufacturer's instructions. The quantity of extracted total RNA was determined using a NanoDrop-1000 Spectrophotometer (Thermo Scientific).

Reverse transcription quantitative PCR (RT-qPCR):

cDNA was synthesised from extracted RNA using the High-Capacity cDNA Reverse Transcription Kit for mRNA/rRNA markers, and the TaqMan® microRNA Reverse Transcription Kit for miRNA markers (Applied Biosystems) following the manufacturer's instructions. A total RNA quantity of 300 ng and 10 ng were used for the reverse transcription reactions of the mRNA and miRNA markers respectively, to ensure there was sufficient amount of RNA to be converted into cDNA.

Body fluid-specific markers suitable for blood identification were selected from the forensic science literature [9, 12-16] along with two reference genes (*18S* and *U6*). Table 1 shows the selected RNA markers for each body fluid. The assays used in this work were designed to amplify only sections of the relevant mRNA molecules, and not the whole transcripts.

Table 1: **The selected TaqMan® Gene Expression Assays and TaqMan® MicroRNA Assays for each body fluid.**

Body fluid	RNA marker	RNA type	Applied Biosystems TaqMan® assay ID	Amplicon length (nt)
Blood	*HBA*	mRNA	Hs00361191_g1	156
	HBB	mRNA	Hs00758889_s1	95
	miR16	miRNA	000391	22
Reference genes	*18S*	rRNA	Hs99999901_s1	187
	U6	snRNA	001973	22

Real-time quantitative PCR was carried out using the TaqMan® Universal PCR Master Mix II Kit, with no AmpErase® UNG (Applied Biosystems) following the manufacturer's instructions for pre-designed primers.

Relative expression ratio (RER):

Similar to the method applied in our previous work on blood samples [5], the relative expression ratio was obtained by dividing the efficiency-corrected C_q values of the less stable RNA marker by the efficiency-corrected C_q values of the more stable RNA marker, or by dividing the C_q values of the body fluid-specific marker by the C_q values of a reference gene, as shown in equations (1) and (2) below:

$$RER = \frac{Cq \ of \ less \ stable \ marker}{Cq \ of \ more \ stable \ marker} \qquad \text{(Equation 1)}$$

$$RER = \frac{Cq \ of \ body \ fluid \ marker}{Cq \ of \ reference \ gene} \qquad \text{(Equation 2)}$$

Statistical analysis:

The data generated from RT-qPCR was analysed using *MxPro* (Agilent Technologies), and *GenEx* statistical software (version 5.4.4) was used for efficiency correction of the raw C_q data. The line graphs and summary statistics were generated using *Microsoft Excel 2016*. *Minitab Express* (version 1.5.0) was used for statistical analysis, including

the Anderson-Darling normality test and one-way analysis of variance (ANOVA) with two different post-hoc analyses (Dunnett and Tukey).

Results:

Expression of RNA transcripts in pure body fluid samples:

Initially, the expression levels of the selected blood-specific markers (*HBA*, *HBB*, and *miR16*) and two reference genes (*18S* and *U6*) were measured in fresh, pure body fluid samples (blood, saliva and semen). The C_q values of each marker in the three body fluid types are recorded in Table 2.

As expected, all blood-specific markers exhibited high expression levels in fresh pure bloodstains. In contrast, neither of the blood-specific mRNA markers (*HBA* and *HBB*) exhibited any expression in fresh pure saliva and semen samples. Only *miR16* showed expression in fresh pure saliva and semen samples. However, when comparing the expression level of *miR16* in blood samples to its expression in saliva and semen samples, the expression level in blood was substantially higher, with average C_q values lower in blood than in saliva and semen by 6.39 and 8.47 cycles, respectively. Based on these findings, the expression of blood-specific markers was not further analysed in aged pure saliva and semen samples, as they showed high or no C_q values in the fresh samples.

With regards to the reference genes, *18S* also exhibited higher expression in fresh pure bloodstains ($C_q = 14.76$) compared to saliva ($C_q = 21.70$) and semen samples ($C_q = 25.88$). *U6* on the other hand, showed a similar expression level in blood and saliva ($C_q = 23.25$ and 22.75 respectively) samples, and low expression in semen samples ($C_q = 30.12$). Both *18S* and *U6* are used as reference genes for mRNA and miRNA studies respectively [9, 16, 17], which means that they should be expressed among all body fluids and tissues at a constant level and should not be affected by experimental conditions. However, the findings of this work suggest otherwise, as the expression of these genes varies between different types of body fluids.

Table 2: The C_q values of blood-specific markers and two reference genes in fresh pure body fluid samples (blood, saliva and semen). n = 8 for each body fluid type.

RNA MARKERS

	Sample	HBA	HBB	miR16	18S	U6
Blood	B1	18.87	19.31	14.46	17.69	23.07
	B11	18.30	16.47	14.94	18.93	22.50
	B2	17.52	22.00	14.30	17.50	22.58
	B22	21.48	20.46	15.03	16.18	23.87
	B3	21.00	17.13	15.22	15.55	24.00
	B33	18.58	18.49	14.96	16.69	22.63
	B4	16.86	15.63	13.74	18.72	23.38
	B44	19.63	23.58	15.41	18.10	23.99
	Mean	**19.03**	**19.13**	**17.42**	**14.76**	**23.25**
Saliva	SV1	No C_q	No C_q	22.57	22.79	22.93
	SV11	No C_q	No C_q	22.06	26.16	23.33
	SV2	No C_q	No C_q	20.93	23.26	22.01
	SV22	No C_q	No C_q	21.53	23.87	22.38
	SV3	No C_q	No C_q	22.62	27.47	23.37
	SV33	No C_q	No C_q	22.52	22.38	22.87
	SV4	No C_q	No C_q	20.38	25.51	22.65
	SV44	No C_q	No C_q	21.00	19.03	22.49
	Mean	**No C_q**	**No C_q**	**23.81**	**21.70**	**22.75**
Semen	SE1	No C_q	No C_q	23.91	24.16	30.27
	SE11	No C_q	No C_q	27.64	27.96	31.81
	SE2	No C_q	No C_q	29.02	26.85	31.05
	SE22	No C_q	No C_q	24.74	26.72	30.29
	SE3	No C_q	No C_q	27.55	25.85	30.12
	SE33	No C_q	No C_q	26.56	26.57	30.13
	SE4	No C_q	No C_q	22.90	23.98	27.31
	SE44	No C_q	No C_q	25.84	25.03	30.01
	Mean	**No C_q**	**No C_q**	**25.89**	**25.88**	**30.12**

Degradation rate of individual RNA transcripts at different ageing time points in pure and mixed bloodstains:

The degradation behaviour of the selected RNA markers was investigated in pure and mixed body fluid samples stored under controlled conditions (*i.e.* room temperature, in a dark, dry place) for up to 60 days.

Figure 1 illustrates the C_q data for each examined RNA marker (*HBA*, *HBB*, *miR16*, *18S* and *U6*) after efficiency correction in pure bloodstains and bloodstains mixed with either saliva or semen. At day 0, in the control samples (fresh pure blood samples), each of the RNAs examined had a different starting expression level. The miRNA marker *miR16* exhibited the highest expression level (lowest C_q value), and *U6* had the lowest expression level (highest C_q value), confirming results that have been published previously [5].

The degradation rate of *HBA* was relatively consistent in pure bloodstains and bloodstains mixed with saliva, however, this marker exhibited slightly lower expression in bloodstains mixed with semen (Figure 1A). The degradation rate of *HBA* in pure bloodstains and mixed samples behaved in a linear manner in the first 30 days of ageing, with the higher C_q values in bloodstains mixed with semen compared to pure bloodstains and bloodstains mixed with saliva being maintained across this time period. At day 60, *HBA* expression level reached a plateau in pure bloodstains and bloodstains mixed with saliva, while in bloodstains mixed with semen the C_q value decreased at 60 days, *i.e.* the expression level of *HBA* marker increased.

The second blood-specific mRNA marker (*HBB*) remained stable across ageing time points in pure bloodstains (Figure 1B), in concordance with previous findings [5]. However, it showed degradation in both mixed bloodstain samples across the first 30 days. At 60 days, the expression level of *HBB* increased (*i.e.* lower C_q values were obtained) in bloodstains mixed with saliva samples, and stabilised at the same level in bloodstains mixed with semen.

The blood-specific miRNA marker (*miR16*) exhibited very interesting behaviour, as it was the only blood-specific marker that remained stable across all ageing time points in all sample types (pure and mixed) with only slight degradation in bloodstains mixed with semen after 30 days of storage (Figure 1C).

When exploring the degradation rate of the reference genes in pure and mixed samples, *U6* remained stable across all ageing time points in all sample types (Figure 1D), with slight degradation at day 30 in bloodstains mixed with semen. In contrast, *18S* exhibited a very similar pattern of gradual degradation in pure bloodstains and in both mixed sample types (Figure 1E).
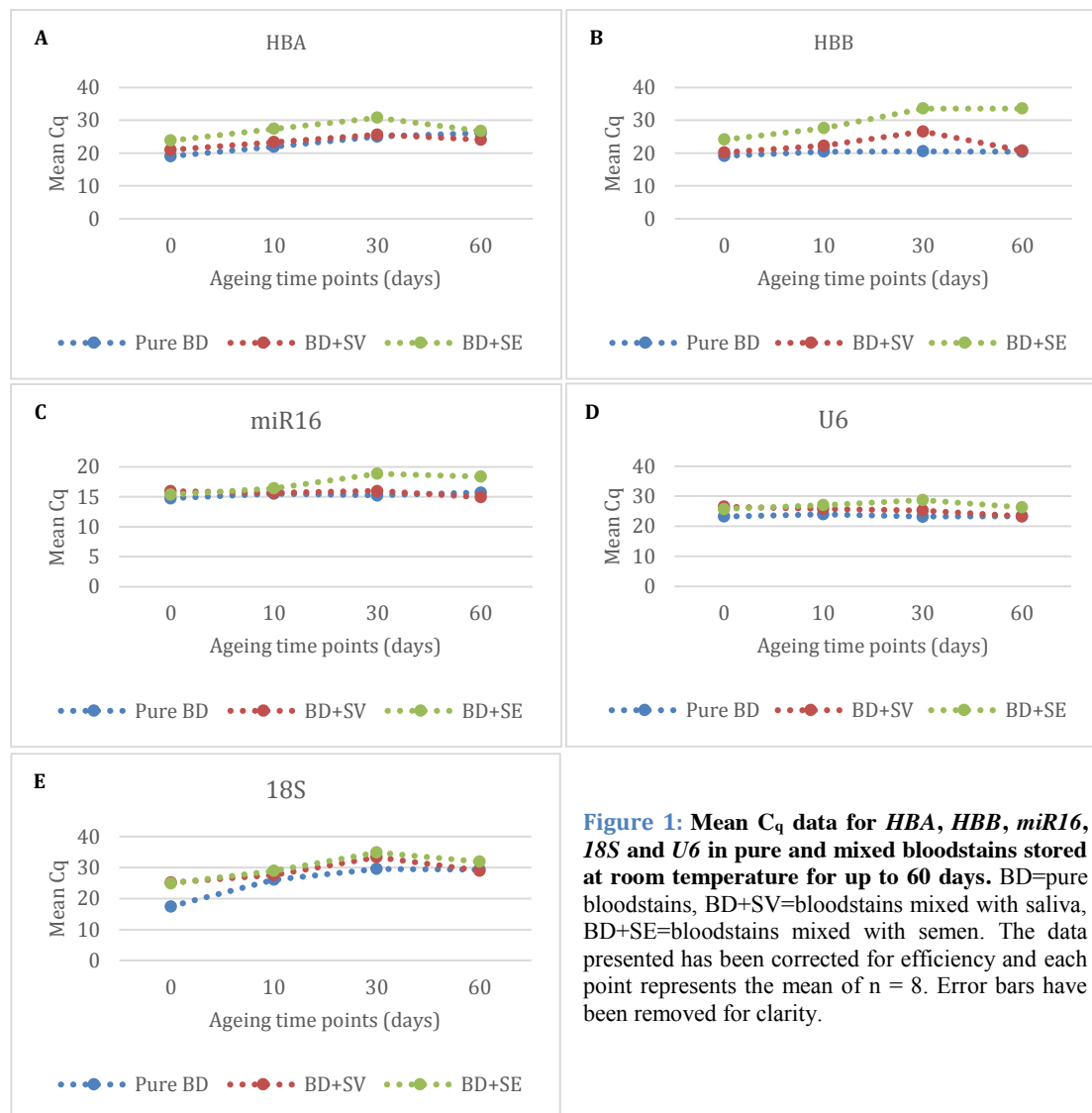

Figure 1: **Mean C$_q$ data for *HBA*, *HBB*, *miR16*, *18S* and *U6* in pure and mixed bloodstains stored at room temperature for up to 60 days.** BD=pure bloodstains, BD+SV=bloodstains mixed with saliva, BD+SE=bloodstains mixed with semen. The data presented has been corrected for efficiency and each point represents the mean of n = 8. Error bars have been removed for clarity.

Statistical analysis:

Statistical analysis was performed on each marker in all different sample types. The Anderson-Darling test of normality showed that all data were normally distributed (P-value > 0.05) with the exception of *HBB* data (P-value = 0.02). Therefore, one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test was applied to the *HBA, miR16, 18S* and *U6* data, and a Kruskal-Wallis test to the *HBB* data, to determine whether any differences were statistically significant.

When comparing mean C_q values across all ageing time points for *HBA* in pure bloodstains and bloodstains mixed with saliva or semen, no significant differences were found (T-value = 0.25, P-value = 0.956 and T-value = 2.16, P-value = 0.103 respectively). Similar results were obtained when comparing mean C_q values for the *miR16* marker in pure bloodstains and bloodstains mixed with saliva (T-value = 0.47, P-value = 0.086) but not when comparing pure bloodstains to bloodstains mixed with semen, where marginally significant differences were identified (T-value = 2.73, P-value = 0.042).

Interestingly, the only blood-specific marker that exhibited significant differences in mean C_q values between pure bloodstains and both mixed sample types was *HBB*. The Kruskal-Wallis test gave P-values of 0.022 and 0.021 (with H-values of 7.65 and 7.68) when comparing pure bloodstains to bloodstains mixed with saliva and pure bloodstains to bloodstains mixed with semen, respectively.

Moreover, the statistical analysis of the reference genes showed no significant differences among different sample types (P-value > 0.05) with the exception of *U6*, which gave a P-value of 0.003 (T-value = 4.47) when comparing its C_q value in pure bloodstains to bloodstains mixed with semen only.

Relative expression ratio (RER):

The relative expression ratio (RER) was applied to determine the relative expression of pairs of RNA markers in pure and mixed bloodstains over time. This approach was used to determine whether mixing bloodstains with other body fluids has an impact on the RER values, and hence on the estimation of bloodstain age. The relative expression of

the less stable RNA marker to the more stable marker across all ageing time points was calculated using the equations as described above, and as used in our previous work [5].

The C_q values for blood-specific markers and the reference genes measured by qPCR at 0, 10, 30 and 60 days were corrected with the determined efficiency of each assay using *GenEx* statistical software (version 5.4.4) (data not shown). One-way analysis of variance (ANOVA) with Dunnett's and Tukey's post-hoc tests were used to compare RER values for pure bloodstains to either bloodstains mixed with saliva or bloodstains mixed with semen. This was carried out in two ways, firstly for data combined across all ageing time points (Dunnett's test), and secondly for each individual ageing time point separately (Tukey's test).

RERs of mRNA to miRNA:

Previously, we showed that the RER of *HBA*/*miR16* was positively correlated with ageing time points, but the RER of *HBB*/*miR16* was not *[5]*. We therefore examined the effect of mixed samples on the RER of *HBA*/*miR16* only. When plotting this measure in the three sample types (Figure 2A), it can be seen that the ratio in pure bloodstains is relatively similar to that in bloodstains mixed with saliva, indicating that the presence of saliva in bloodstains did not affect the *HBA*/*miR16* ratio. However, this is not the case in bloodstains mixed with semen, as the ratio started with a higher value than in pure bloodstains (1.56) and then over time dropped below this (1.45) at 60 days.
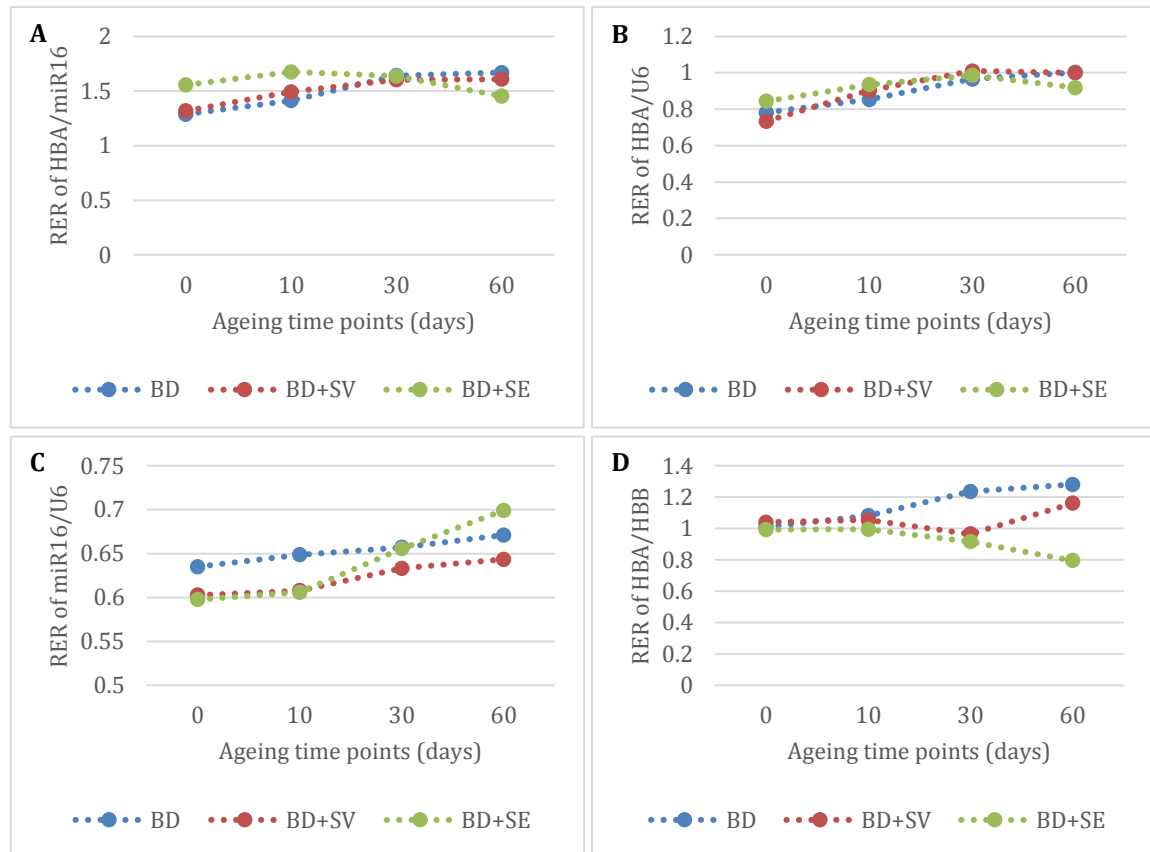
Figure 2: Mean RER of blood-specific RNA markers in pure and mixed bloodstains stored at room temperature for up to 60 days. RER of (A) *HBA/miR16*, (B) *HBA/U6*, (C) *miR16/U6*, and (D) *HBA/HBB*. BD=pure bloodstains, BD+SV=bloodstains mixed with saliva, BD+SE=bloodstains mixed with semen. Each point represents the mean of n = 8. Error bars were omitted for clarity.

The differences between the RERs of *HBA/miR16* in pure and mixed sample types were statistically evaluated. When applying the Anderson-Darling normality test, all the data were determined to be normally distributed (P-value > 0.05), therefore parametric analysis was used. One-way analysis of variance (ANOVA) with Dunnett's and Tukey's post-hoc tests were used to compare the RER values of *HBA/miR16* in pure bloodstains to either bloodstains mixed with saliva or bloodstains mixed with semen.

For data combined across all ageing time points, there were no significant differences between the RERs of *HBA/miR16* in either mixed sample type when compared with pure bloodstains (Figure 3A: T-value = 0.02, P-value = 0.999 for pure bloodstains versus bloodstains mixed with saliva, and T-value = 0.77, P-value = 0.674 for pure bloodstains versus bloodstains mixed with semen).

However, when comparing the ratio in pure bloodstains to mixed samples at each specific ageing time point, the RER of *HBA/miR16* was significantly different between

pure bloodstains and bloodstains mixed with semen in fresh samples and samples aged for 10 days (T-value = 3.37, P-value = 0.02, and T-value = 3.66, P-value = 0.02 respectively), but not at other ageing time points or in bloodstains mixed with saliva at any ageing time point (P-value > 0.05).

RERs of mRNA and miRNA to U6:

Similarly, the RERs of *HBA/U6* and *miR16/U6* have previously been shown to exhibit a significant positive correlation with ageing time points [5]. When plotting the RERs in the three sample types over time (Figure 2B), there are minimal differences in the RER of *HBA/U6* between pure and mixed bloodstains, indicating that the presence of saliva or semen in bloodstains did not substantially affect this ratio. However, the RER of *miR16/U6* showed some differences when comparing pure bloodstains to mixed bloodstains (Figure 2C). In the first 10 days, this ratio was lower in mixed samples compared to pure bloodstains. After 10 days of storage, the RER values increased gradually in bloodstains mixed with saliva, and increased rapidly in bloodstains mixed with semen.
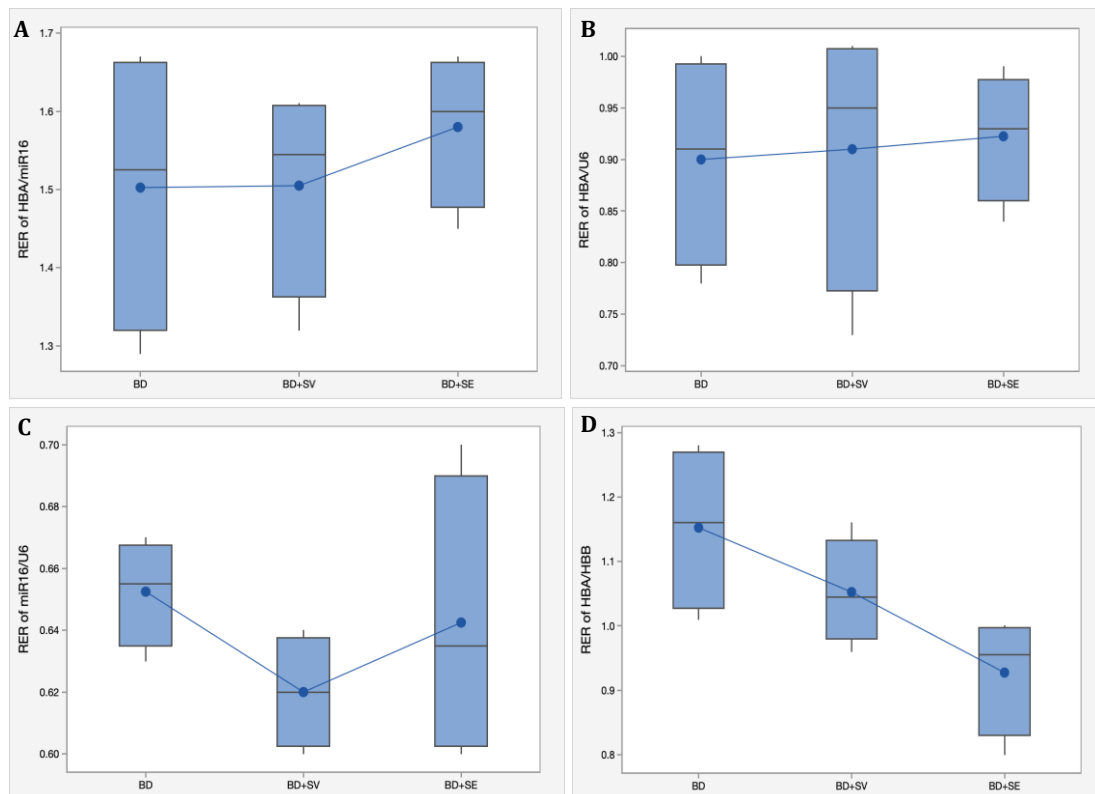

Figure 3: **Mean RER of blood-specific RNA markers in pure and mixed bloodstains stored at room temperature across all ageing time points. RER of** (A) *HBA/miR16*, (B) *HBA/U6*, (C) *miR16/U6*, and (D) *HBA/HBB*. BD=pure bloodstains, BD+SV=bloodstains mixed with saliva, BD+SE=bloodstains mixed with semen. Each point represents the mean of n = 32.

The differences between the RERs of *HBA/U6* and *miR16/U6* in in pure bloodstains versus mixed stains were statistically analysed, both for data combined across all ageing time points, and for each individual ageing time point separately. The data were found to be normally distributed (P-value > 0.05) when applying an Anderson-Darling normality test, so again one-way ANOVA with Dunnett's and Tukey's post-hoc tests was used.

For data combined across all ageing time points, there was no difference in the RER of *HBA/U6* between pure bloodstains and either of the mixed samples (Figure 3B, T-value = 0.14, P-value = 0.986 for pure bloodstains versus bloodstains mixed with saliva, and T-value = 0.31, P-value = 0.932 for pure bloodstains versus bloodstains mixed with semen). There was a notable difference in the RER of *miR16/U6* between pure bloodstains and mixed bloodstains, however no significant difference was recorded (Figure 3C, T-value = -0.46, P-value = 0.858 for pure bloodstains versus bloodstains mixed with semen, and T-value = -1.51, P-value = 0.274 for pure bloodstains versus bloodstains mixed with saliva). When comparing the values of the RERs for both *HBA/U6* and *miR16/U6* in pure bloodstains versus mixed samples at each specific ageing time point separately, no significant differences were detected (all P-values > 0.05).

RERs of mRNA markers:
Finally, the effect of body fluid mixtures on the RER of *HBA/HBB* was examined. Figure 2D shows the RERs of *HBA/HBB* in the three sample types. As expected, in pure bloodstains the ratio increased in a linear fashion with increasing ageing time points. However, this is not case in mixed samples, as the ratio fluctuates in bloodstains mixed with saliva samples and decreased over time in bloodstains mixed with semen samples.

One-way ANOVA with Dunnett's and Tukey's post-hoc tests was carried out to compare the RER values of *HBA/HBB* in pure bloodstains to bloodstains mixed with saliva and bloodstains mixed with semen, both for data combined across all ageing time points, and for each individual ageing time point separately. For data combined across all ageing time points, a significant difference was found when comparing pure bloodstains to bloodstains mixed with semen (Figure 3D, T-value = -3.09, P-value =

0.023), despite the different patterns observed in the RER of *HBA/HBB* in pure bloodstains and mixed samples. In contrast, no significant difference was found when comparing the same ratio in pure bloodstains to bloodstains mixed with saliva (Figure 3D, T-value = -1.37, P-value = 0.330).

When comparing pure bloodstains versus mixed samples at each ageing time point separately, the RER of *HBA/HBB* was significantly different (T-value = -5.56, P-value < 0.0001) between pure bloodstains and bloodstains mixed with saliva in samples aged for 30 days, and between pure bloodstains and bloodstains mixed with semen in samples aged for 30 and 60 days (T-value = -6.55, P-value < 0.0001, and T-value = -9.86, P-value < 0.0001 respectively).

Discussion:

Biological samples that are found at crime scenes are commonly found as mixtures, where two or more body fluids are mixed together, either from the same individual or multiple individuals. The difficulties associated with analysing samples of this type can be addressed by applying a method using body fluid-specific markers both to identify body fluid type and to estimate the age of the stain simultaneously.

The findings of our previous work [5] have shown that the RERs of blood-specific markers can be considered as a potential method for estimating the age of bloodstains. However, it is important to investigate whether there is any limitation to this proposed method for estimating the time since deposition as a result of body fluids being present in mixed stains. The main aim of this research was therefore to evaluate the impact of mixing bloodstains with other body fluid types (saliva and semen) on the RER values of various blood-specific markers, and hence the effect on the estimation of bloodstain age or time since deposition.

In this work, pure bloodstains and bloodstains mixed with saliva or semen were stored for up to 60 days under controlled conditions (*i.e.* room temperature, in a dark, dry place), and at each desired ageing time point, total RNA was extracted and RNA analysis was performed. The expression level of multiple blood-specific markers (*HBA*, *HBB* and *miR16*) along with two reference genes (*18S* and *U6*) was quantified using RT-qPCR in all different sample types (pure and mixed). Blood-specific markers

showed low or no expression in the other body fluids (saliva and semen), confirming that the selected markers are truly blood-specific. These findings are in accordance with the EDNAP collaborative exercise [18] and many other studies [19-22] that have investigated the expression of blood-specific markers in different body fluids, where no expression of *HBA* and *HBB* has been found in saliva and semen samples.

When analysing the degradation rate of the reference genes, only *U6* showed a relatively constant level in all sample types across ageing time points, with evidence of slight degradation at day 30 in bloodstains mixed with semen. In contrast, *18S* did not remain stable across ageing time points in any sample type, which is in agreement with previous studies that have found that the level of *18S* did not remain constant in aged body fluid samples [23, 24]. This likely to be due the degraded status of the aged samples analysed here, which were set up to mimic the difficult nature of the types of biological samples that are commonly found at crime scenes. This indicates that although *18S* might be a suitable reference gene for gene expression studies in clinical samples, its utility as a reference gene for forensic purposes may be limited.

The RT-qPCR data for the blood-specific markers demonstrates that each RNA transcript showed a unique pattern of degradation behaviour in pure and mixed bloodstains. *HBA* was the only blood-specific marker which exhibited no significant differences in degradation behaviour between all samples types, while the degradation rate of *HBB* degradation was significantly different in all samples types, and *miR16* was significantly different between pure bloodstains and bloodstains mixed with semen (but not saliva). This pattern indicates that the presence of saliva or semen in bloodstains can affect the expression level of blood-specific mRNA and miRNA markers.

The RERs of different combinations of blood-specific markers were calculated for each sample type at each of the ageing time points: *HBA/miR16*, *HBA/U6*, *miR16/U6*, and *HBA/HBB*. All four of these ratios were affected to some extent by the mixing of bloodstains with other body fluids, as none of the RERs showed exactly the same pattern over time in pure and mixed bloodstains, although the differences were minimal in many cases. Statistical evaluation of these differences revealed that when data was combined across all ageing time points, the only significant difference detected was for the comparison of the *HBA/HBB* ratio between pure bloodstains and bloodstains mixed

with semen, but not for any other comparison. This is likely to be due to the degradation behaviour of *HBB*, which exhibited the most substantial differences in C_q values between pure and mixed bloodstains.

When examining these patterns in more detail by statistically evaluating the differences in RERs at individual ageing time points separately, no significant differences were detected when comparing the *HBA/U6* and *miR16/U6* ratios between pure and mixed bloodstains at any ageing time point. In contrast, there was a significant difference in the *HBA/miR16* ratio between pure bloodstains and bloodstains mixed with semen at 0 and 10 days of ageing. In addition, there was a significant difference in the *HBA/HBB* ratio between pure bloodstains and bloodstains mixed with saliva at 30 days of ageing, and between pure bloodstains and bloodstains mixed with semen at 30 and 60 days of ageing. As above, the degradation behaviour of *HBB* in mixed stains seems to be responsible for the majority of the differences in these ratios, suggesting that it may be preferable to avoid the use of this transcript when it is suspected that biological samples are mixed.

We thus observed differences of varying magnitudes in these four ratios between pure and mixed bloodstains, a small number of which were shown to be statistically significant. It should also be taken into consideration that the relatively small sample sizes used in this study mean that we may only be able to detect significance for relatively large effects. Some of the smaller, non-significant differences that we have observed may therefore also be important, and this lack of statistical significance should be interpreted with caution. Our findings therefore indicate that the presence of body fluid mixtures may have an impact on the RERs of some blood-specific RNA markers, and thus it is important to consider this when the presence of mixed stains is suspected. However, our data also indicate that some blood-specific markers and their RERs are affected to a much lesser extent than others by the presence of mixed body fluid stains, for example the *HBA/U6* and *miR16/U6* ratios. The calculation of these ratios may therefore still be of use in the estimation of the age of bloodstains, even if the blood is present in a mixture with saliva or semen. However, to determine how applicable this is across different body fluids, more mixtures should be studied, including sample types such as menstrual blood, vaginal secretions, urine and sweat, as well as mixtures of more than two body fluids. Another potential avenue of research would be to examine

different volume ratios of body fluid mixtures, as this study only considered equal volume mixtures of blood with other body fluids. Additionally, there are environmental factors that might affect the RERs of body fluid-specific markers that have not been explored as part of this project, such as UV exposure, humidity and temperature.

Conclusion:

The main outcome of this work is that mixing bloodstains with other types of body fluids has a significant impact on some RERs of blood-specific markers, but not on others. The potential use of some of these RERs as a method for estimating the age, or time since deposition, of bloodstains is therefore not limited by the fact that forensic samples are frequently found as mixtures of more than one body fluid type.

Conflict of interest

None.

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